Identification of Endogenous Electrophiles by Means of Mass Spectrometric Determination of Protein and DNA Adducts

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Monitoring exposure to alkylating agents may be achieved by quantitatively determining the adduct levels formed with nucleic acids and/or proteins. One of the most significant results arising from the application of this approach has been the discovery in control populations of "background" levels of alkylated nucleic acid bases or alkylated proteins, in particular hemoglobin (Hb). In the case of Hb, a wide variety of such adducts have been detected and quantitated by mass spectrometric techniques, with methylated, 2-carboxyethylated, and 2-hydroxyethylated modifications being most abundant. Although the source of these alkylation products is unknown, both endogenous and exogenous sources may be proposed. We have recently confirmed the presence of the N-terminal hydroxyethylvaline adduct in control human Hb using tandem mass spectrometry (MS–MS) and have now established background levels using GC–MS in more than 70 samples. Smoking raises the levels of the adduct up to 10-fold and occupational exposure to ethylene oxide up to 300-fold.

Background levels of alkylated nucleic acids may be studied by analysis of N7-alkylated guanine or N3-alkylated adenine, which are excised from nucleic acids after their formation and are excreted in urine. Although the presence of some of these urinary constituents may be accounted for by their natural occurrence in RNA or diet, the endogenous or exogenous source of others is unknown. Quantitative methods using MS–MS have now been developed for five of the observed urinary alkylguanines [N7-methyl-, N²-methyl-, N²-dimethyl-, N7-(2-hydroxyethyl)-, and N²-ethylguanine]. A GC–MS method has also been developed to measure urinary thymine glycol as a possible monitor of oxidative DNA damage.

Introduction

A variety of analytical approaches have been and are being developed to detect carcinogen adducts in DNA as a means of monitoring the biologically effective dose of the carcinogen received by an individual. These detection methods include ^{32}P-postlabeling, fluorescence spectroscopy, radio- and enzyme-linked immunoassay, and MS procedures. All of these methods are extremely sensitive, the highest detection limit being with ^{32}P-postlabeling which can show the presence of one carcinogen-modified nucleic acid base per 10^{10}–10^{11} bases. Mass spectrometry procedures, although not as sensitive as postlabeling, confer the additional advantage that they give chemical structural information on the adduct. Mass spectrometry has also been widely used for the detection of carcinogen adducts with proteins, and in particular with hemoglobin (Hb). The ready availability of Hb, coupled with its long lifetime, makes it a convenient molecule for monitoring acute and chronic carcinogen exposure.

One of the most significant findings in the studies of DNA and Hb carcinogen adducts has been the detection of "background" levels of adducts in supposedly unexposed populations. The source of these background adducts is in general unknown, and could be due to one or more of the following: *a*) an endogenous metabolic pathway, *b*) an endogenous catabolic pathway that produces the adduct as a degradation product of another molecule, *c*) exogenous exposure to the carcinogen through a route or mechanism that was not recognized, *d*) exposure to the carcinogen produced by an endogenous mechanism, *e*) intake of the adduct through the diet, or *f*) contamination of reagents/laboratory environment with the carcinogen.

In this paper the background adducts that have been detected by MS are reviewed, and our current approaches for increasing specificity for their detection are outlined. Most of the adducts to be discussed are those formed by the reaction between electrophilic carcinogens and nucleophilic residues within the macromolecule.

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Hemoglobin Adducts

Methyl

S-Methylcysteine (Fig. 1) was the first adduct shown to have background levels (*1*). It was detected by chemical ionization GC–MS analysis of Hb acid hydrolysates. Its concentration in Hb is species dependent, being highest in avian species (e.g., 296 nmole/g globin in chickens). The lowest level recorded is in the hamster (5.6 nmole/g globin). Humans contain about three times the adduct present in hamsters (Table 1). Although the identification of S-methylcysteine is without doubt, its source is uncertain.
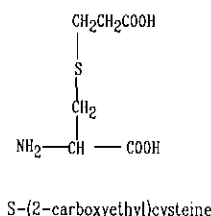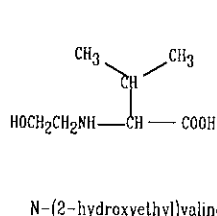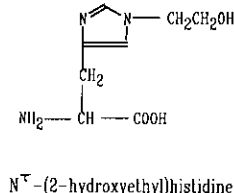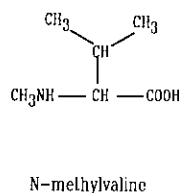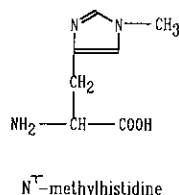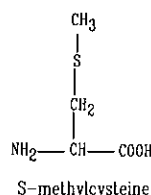

FIGURE 1.   Structures of some alkylated amino acids isolated from control hemoglobin.

Table 1. Background levels of modified amino acids in human hemoglobin.

Amino acid	Adduct level, nmole/g globin	Reference
S-Methylcysteine	16.4	Bailey et al. (*1*)
N⁷-Methylhistidine	12–42	Törnqvist et al. (*6*)
N⁷-Methylvaline	0.5	Törnqvist et al. (*6*)
S-(2-Carboxyethyl)cysteine	8.5	Bailey et al. (*19*)
N-(2-Hydroxyethyl)histidine	1.6	Van Sittert et al. (*10*)
S-(2-Hydroxyethyl)cysteine	1–3	Kautiainen et al. (*16*)
N-(2-Hydroxyethyl)valine	0.05	Bailey et al. (*13*)
Sulfinamides	pmole/g Hb	
4-Aminobiphenyl	0.18	Bryant et al. (*22*), Perera et al. (*23*)
Aniline	41	Stilwell et al. (*24*)
o-Toluidine	0.32	Stilwell et al. (*24*)
m-Toluidine	6.4	Stilwell et al. (*24*)
p-Toluidine	0.65	Stilwell et al. (*24*)
2-Aminonaphthalene	0.04	Stilwell et al. (*24*)

Artifactual production during the work-up procedure is conceivable (*2*), although this hypothesis has not been supported by our work (*3*). S-Methylcysteine sulfoxide, which may be a precursor of S-methylcysteine, is a natural constituent of food (*4*), and the free amino acid has also been detected in plants (*5*). Incorporation of the methylated amino acid in the biosynthesis of Hb has been proposed by Törnqvist et al. (*6*) as a contributor to the background levels of S-methylcysteine. Endogenous pathways involving methylation by S-adenosylmethionine are also feasible.

Human control Hb also contains N⁷-methylhistidine (detected by GC–MS or amino acid analysis) (Fig. 1; Table 1) (*6*). This is also a constituent of food and could be misincorporated into Hb. Additionally, it is a constituent of muscle proteins and exists in human urine (*7*). The N-terminal valine in Hb was shown by Törnqvist et al. (*6*) to be partially methylated (Fig. 1; Table 1). The misincorporation of N-methylvaline in Hb biosynthesis was not considered to be likely, and endogenous methylation by S-adenosylmethionine or by a reaction with formaldehyde was proposed as the source of Hb N-methylvaline.

The levels of background methylated adducts in Hb prevent the use of these adducts as monitors of low levels of exogenous methylating agent exposure. Thus, using the rat as a model, to generate a 50% increase over background levels of S-methylcysteine would require the animal to be administered about 20 mg nitrosodimethylamine/kg body weight (*1*). Exposure to stable isotope-labeled methylating agents has, however, satisfactorily been monitored (as there will naturally be no background adducts for these) through determinations of Hb S-methylcysteine (*8*) or carboxylic acid methyl esters (*9*).

2-Hydroxyethyl

N⁷-(2-Hydroxyethyl)histidine (Fig. 1) in Hb may be determined by GC–MS following total acidic hydrolysis of the globin (6 N HCl, 110°, *in vacuo*), ion exchange chromatography, and derivatization. Background levels of 1.59 ± 0.18 nmole/g globin were found in man (*10*) and 1.3 - 2.8 nmole/g globin in rats (*11*). The analytical procedure involves time-consuming chromatographic steps and detection limits are only about 0.5 nmole/g globin.

Hydroxyethylation of the NH_2-group of the N-terminal valine has also been demonstrated in control populations. This adduct (Fig. 1) may be determined following a modified Edman degradation (using pentafluorophenyl isothiocyanate) to liberate it from the protein as a thiohydantoin (*12*). The product is quantitated by GC–MS using globin modified by d_4-ethylene oxide as internal standard. The procedure used in our laboratory involves conversion of the thiohydantoin to its trimethylsilyl (TMS) derivative, followed by analysis by electron impact (EI) GC–MS (*13*). The detection limit is 10 pmole adduct/g globin. In our original study, background levels of 52.1 ± 20.5 pmole N-(2-hydroxyethyl)valine (HEV)/g globin were found in control subjects ($n = 23$). We have subsequently analyzed a further 47 controls. HEV was found in every person (range 13–126 pmole/g globin), with a mean value of 46.4 ± 26.1 pmole/g globin. No correlations have been found with age or sex.

The relative significance of the HEV background levels may be judged by comparison with what we have observed in exposed individuals, e.g., patients receiving a single dose of the anti-
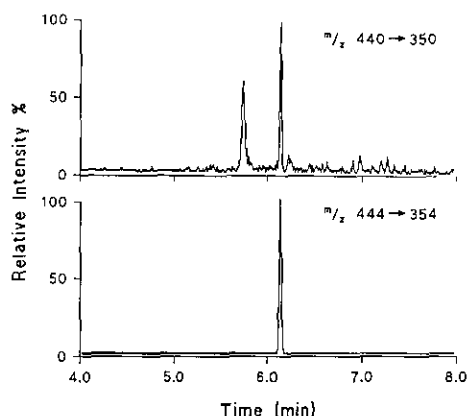
FIGURE 2. Multiple reaction monitoring GC–MS of the trimethylsilyl derivative of *N*-(2-hydroxyethyl)valine pentafluorophenylthiohydantoin (m/z 440 to 350) and of its tetradeuterated analog used as internal standard (m/z 444 to 354). The retention time of this derivative is 6.2 min. The sample was obtained from control human globin.

cancer drug 1-[4-amino-2-methyl(pyrimidin-5-yl)methyl]-3-(2-chloroethyl)-3-nitrosourea (ACNU): 330 pmole/g globin (*14*); smokers of 10 cigarettes/day: 170 pmole/g globin (*13*); ethylene oxide workers: up to 16.1 nmole/g globin (*15*).

Further validation of the chemical nature of the background adduct has been achieved by using MS–MS/tandem mass spectrometry for detecting the product as it eluted from the GC. The MS–MS instrument used to determine this was a VG-70 SEQ, which has the configuration:electrostatic analyzer, magnet, R_f-only quadrupole collision cell, quadrupole analyzer. The molecular ion of the TMS derivative of HEV pentafluorophenyl-thiohydantoin (m/z 440) fragments in the collision cell to yield a fragment (or daughter) ion (m/z 350), which is detected by the quadrupole analyzer. Monitoring this fragmentation (multiple reaction monitoring; MRM) yields a much more selective detection of the compound in comparison to single MS selective ion recording (SIR) of m/z 440. Contaminants that are detected on a SIR trace because they yield (like HEV) an ion at m/z 440 are virtually absent from an MRM trace. Quantitation may be achieved in the MS–MS analysis by rapid switching from the fragmentation 440 to 350 to the analogous fragmentation derived from the tetradeuterated internal standard 444 to 354, and determination of peak area ratios. We have achieved satisfactory calibration lines for such an analysis and have applied it to globin samples from a population exposed occupationally to ethylene oxide and to controls (unpublished observations). Figure 2 shows the MRM analysis for HEV in control globin, which clearly shows detectable levels of endogenous HEV.

Numerous speculations may be made as to the source of HEV. It is not incorporated into Hb (*16*), and therefore a dietary source seems unlikely. It seems likely that endogenous or exogenous exposure to a hydroxyethylating agent is involved. Possible sources are environmental ethylene or ethylene oxide (exhaust fumes, cigarette smoke), or endogenously produced ethylene. Evidence has been obtained that lipid peroxidation and metabolism of intestinal bacteria may both contribute to HEV levels in Hb (*17,18*).

2-Carboxyethyl

The discovery of the presence of S-(2-carboxyethyl)cysteine (Fig. 1) in hydrolyzed globin stemmed from an investigation into

the binding of acrylamide to Hb (*19*). Acrylamide has the highest binding index known (8.6 nmole adduct/g globin per μmole/kg body weight dose [rat]) for reaction with Hb and is believed to produce S-(3-amino-3-oxopropyl)cysteine. During the acid hydrolysis of the protein for adduct isolation, the carboxamido group hydrolyzes to the acid, yielding S-(2-carboxyethyl)cysteine (CEC), the determination of which was used for GC–MS monitoring of acrylamide exposure. Very high background levels of CEC were found in hydrolyzed Hb of rats (21.4 nmole/g globin) and man (Table 1). It is conceivable that these levels are caused by exogenous exposure to acrylamide, which is widely used in the production of polyacrylamides. In the rat, the dose (IP) of acrylamide needed to generate the background may be estimated as 0.3 mg/kg (*19*). CEC is a natural constituent of human urine (*20*), and endogenous pathways for its formation from components of the glycolytic pathway may be feasible. However, it should be made clear that the chemical nature of the modified cysteine in intact Hb has not yet been determined.

Phenylhydroxyethyl

In view of our success at detecting hydroxyethyl adducts, we have recently devised methods for monitoring phenylhydroxyethyl adducts that would be derived from exposure to styrene oxide (*21*).

An analytical method for the N-terminal valine adduct has been developed using the modified Edman degradation (see "2-Hydroxyethyl"). The resulting pentafluorophenylthiohydantoin is purified by Sep-Pak solid-phase chromatography, converted to the acetyl or TMS derivative, and detected by GC–MS SIR. Quantitation is achieved using an internal standard prepared by reacting d_8-styrene oxide *in vitro* with Hb. The lower limit of detection of the assay is 10 pmole adduct/g globin, and the yield of thiohydantoin produced in the procedure (determined by radiochemical means) accounted for 5.2% of total globin alkylation *in vitro* by styrene oxide.

Styrene oxide also reacts with carboxylic acid residues in Hb, yielding phenylhydroxyethyl esters. Mild basic hydrolysis of the globin cleaves these esters, yielding 1-phenyl-1,2-ethanediol (styrene glycol). The yield of this product extracted into ethyl acetate accounted for 15% of total globin alkylation *in vitro* by styrene oxide. A GC–MS SIR method to detect styrene glycol in globin hydrolysates, with a limit of detection of 20 pmole/g globin, has been developed to yield a second estimate of the bound dose of styrene oxide. The liberated styrene glycol is converted to its TMS derivative and quantitated by SIR using d_8-styrene glycol (derived from globin labeled *in vitro* with d_8-styrene oxide) as internal standard. Rigorous solvent purification was required to remove contaminants. Analysis of globin samples ($n = 10$) from unexposed humans did not reveal the presence of background levels of styrene oxide adducts, with the above-mentioned limits of detection.

Aromatic Amines

Aromatic amine adducts with Hb may be determined by acidic or alkaline hydrolysis of the labile cysteine sulfinamide adducts, followed by extraction of the free amine, derivatization, and GC–MS SIR. Studies on 4-aminobiphenyl (4-ABP) adducts by Bryant et al. (*22*) showed the presence of species-dependent

background levels. Human background levels were 28 pg/g Hb, whereas rats had 500–3000 pg/g Hb, and monkeys and fish had less than the detection limit of 5–10 pg/g Hb. The background in humans was also reported by Perera et al. [32.2 ± SD 12.3 pg/g Hb (*23*)]. The source of this background level is unlikely to be endogenous, and it is suspected to be from passive cigarette smoking, diet, or air pollution. Analogous analyses for adducts from other aromatic amines (Table 1) also showed background levels, again of an unknown source (*24*).

We have recently developed methods for determining adducts to Hb of 4,4'-methylenedianiline (MDA) (*25*) and 4,4'-methylene-*bis*(2-chloroaniline) (MOCA) (*26*). These both involve basic hydrolysis of Hb (which liberates 40% of the bound dose from its sulfinamide adduct), solvent extraction, conversion to the pentafluoropropionyl derivative, and EI GC–MS SIR, using a deuterated internal standard. The limit of detection is 10 pmole/g Hb for MDA and 20 pmole/g Hb for MOCA. In the case of MDA, a second adduct, containing the acetylated amine, was also detected and similarly determined. Analysis of rat samples

showed no detectable background of MDA, *N*-acetyl MDA, or MOCA. Analyses have been conducted on MDA Hb samples obtain from humans. Adducts were not detected in 10 control subjects, but were found in 12 workers with occupational exposure to MDA (up to 55.3 pmole MDA adduct/g globin and 91.8 pmole *N*-acetyl MDA adduct/g globin).

Nucleic Acid Adducts

Urinary Alkylated Purines

Modification of nucleic acids by alkylating carcinogens results in the formation of many adducts, including N7-alkylguanines and N3-alkyladenines. Both of these bases are rapidly removed from the nucleic acid and excreted in the urine. Their measurement represents an approach for screening an individual's exposure to alkylating carcinogens over the previous 24 hr. However, background levels, particularly of low molecular weight alkyl groups do exist.

N7-Methylguanine (Fig. 3) is abundant in urine (about 6.5 mg/24 hr) (*27*). Our method for this purine's determination involves Sep-Pak column chromatography, followed by conversion to the pentafluorobenzyl N-heptafluorobutyryl derivative, and EI GC–MS SIR. Because of the high background levels of N7-methylguanine, this method is unsuitable for monitoring human methylating agent exposure except in cases where the compound is stable-isotope labeled, for which there would be no background (*28*). The source of N-7-methylguanine is tRNA, in which it is a minor base. We have also monitored urinary N3-methyladenine (Fig. 3) by GC–MS SIR of its *tert*-butyldimethylsilyl derivative (TBDMS) (*29*), and found this also to be present in human urine, although at much lower levels than N7-methylguanine (4.5–16.1 µg/24 hr). The source of N3-methyladenine was shown to be largely due to the diet by Prevost et al. (*30*).

We have now used MS–MS to explore the presence of other alkylguanines in urine. Aliquots of urine were applied to a C-18 Sep-Pak column, which was washed with water, and the alkylguanine fraction was eluted with aqueous methanol. Parent ion scanning of m/z 151 (guanine$^+$) (*31*) showed the presence of
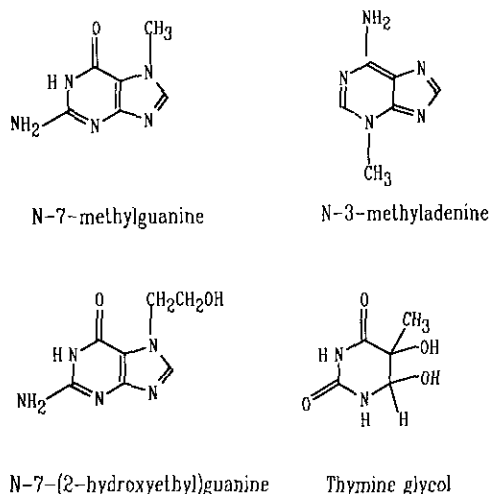
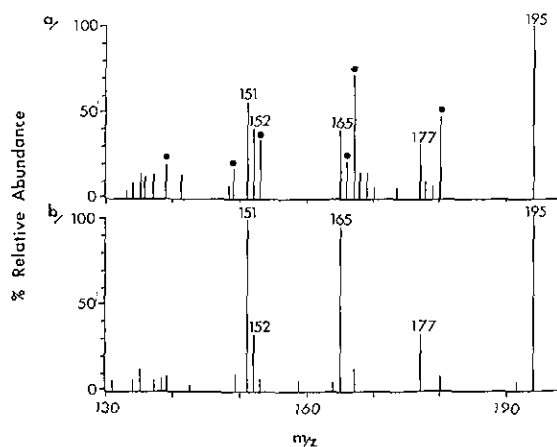FIGURE 3. Structures of some modified nucleic acid bases isolated from urine.

N-7-methylguanine

N-3-methyladenine

N-7-(2-hydroxyethyl)guanine

Thymine glycol

FIGURE 4. Electron impact daughter ion spectra of m/z 195 (collision energy ~ 30 eV, air in the gas cell ~ 10^{-8} mbar). (*a*) Human urine eluted from Sep-Pak cartridge with 70% aqueous methanol. Ions marked (●) correspond to ions observed in a solvent blank taken through the method or contaminants in the urine. (*b*) Same urine sample spiked with N7-(2-hydroxyethyl)guanine.
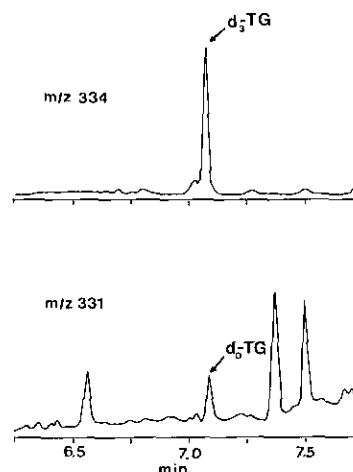
FIGURE 5. Selective ion recording GC–MS of the *tert*-butyldimethylsilyl derivative of thymine glycol (TG) (m/z 331) and of its trideuterated analog used as internal standard (m/z 334). The retention time of the derivative is 7.1 min. The sample was obtained from control human urine.

several ions, notably one at m/z 179 and one at m/z 195, isobaric with an ethylated (or dimethylated) and a hydroxyethylated guanine, respectively.

An EI daughter spectrum of m/z 179 tentatively revealed the presence of both N^2-dimethylguanine and an ethylated guanine. By performing hydrogen/deuterium exchange it was possible to distinguish N^2-dimethylguanine (which has three exchangeable positions) from ethylated guanine isomers (which have four exchangeable positions) (32). A combination of EI and fast atom bombardment (FAB) daughter ion scanning on deuterium-exchanged material confirmed the presence of N^2-ethylguanine. Evidence has been obtained to suggest that the constituent of m/z 195 is N7-(2-hydroxyethyl)guanine (Fig. 3). Daughter ion scans of m/z 195 on urine fractions eluted with 70% aqueous methanol from a Sep-Pak column yielded ions consistent with this structure (Fig. 4).

Further investigation of urinary alkylpurines was carried out using GC–MS and GC–MS–MS. Samples were partially purified by Sep-Pak chromatography and in some cases HPLC, and converted to their TBDMS derivatives. The presence of N7-methyl-, N^2-methyl-, N^2-dimethyl-, N^2-ethyl- and N7-(2-hydroxyethyl)-guanine in urine was confirmed by GC–MS SIR. Deuterated analogs of these five alkylguanines were synthesized and are being used as internal standards for quantitation by GC–MS using SIR or GC–MS–MS using MRM. FAB MS–MS has also been used (on underivatized samples) as further structure confirmation.

N7-(2-hydroxyethyl)guanine could just be detected by SIR (maximum levels 3 μg/24 hr) but not by the less sensitive technique of MRM. Its presence is of interest as it is unlikely to be a tRNA component and probably represents exogenous or endogenous exposure to a hydroxyethylating agent. N^2-Methyl and N^2-dimethylguanine are both derived from tRNA, but the source of N^2-ethylguanine is unknown.

Urinary Thymine Glycol

A further type of damage that occurs in DNA is caused by active oxygen species. Hydroxyl radicals produce modification of all four DNA bases and measurement of the extent of formation of these may be used as an indication of exposure. One example, cis-thymine glycol (TG) (Fig. 3), has been detected in urine by HPLC (33). We have now developed a GC–MS assay for TG, in which it is quantitated using a d_3-labeled internal standard. The procedure involves charcoal extraction (to remove contaminants), chromatography on a boronate affinity column, and conversion of TG to its TBDMS derivative. Satisfactory calibration lines suitable for the detection of 1 ng TG/mL urine have been generated. A representative SIR trace is shown in Figure 5. Preliminary values for background levels in urine are 0–750 pg/mL.

Conclusion

This review has dealt only with MS methods for detecting background adducts and has concentrated on those formed by low molecular weight alkylating agents. It should be pointed out that MS evidence also exists for the presence of benzo[a]pyrene adducts in placental DNA from nonsmokers (34), and there are many studies where benzo[a]pyrene adducts have been detected by other techniques both in DNA (35) and in serum protein (36).

At present it is impossible to draw firm conclusions as to the source of most background adducts. However, the presence of benzo[a]pyrene or aromatic amine adducts would clearly indicate exogenous exposure to the chemicals. For the lower molecular weight adducts, both exogenous and endogenous exposure are likely to be involved, hydroxyethylation being of particular interest owing to its presence in both Hb and nucleic acids. For methylated adducts, their endogenous generation or dietary uptake is so high that it is not possible to determine exogenous sources of methylating agent exposure, except under special circumstances such as dietary control (30). However, except in the cases where background adducts are incorporated intact from an exogenous source (e.g., diet), their presence must indicate a background carcinogen risk, against which the relative risk associated with environmental exposure to carcinogens must be assessed.

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